between 40-150 pN (500 nm/s pulling speed) which were evident as "saw tooth" peaks in force spectra. Spectra with up to 11 peaks were obtained. The curves also revealed region(s) of very low stability that could be stretched >100 nm prior to any unfolding event. In force extension curves of a shorter three domain construct comprised only of the M-domain and its two flanking Ig domains (i.e., C1-M-C2) an extensible region with a contour length of 43 ± 4 nm and persistence length of 0.42 ± 0.1 nm was also observed before domain unfolding in no more than two sawtooth peaks. The latter data suggest that the M-domain contributes to the extensible segment of cMyBP-C and that the M-domain exhibits mechanical properties that are distinct from those of Ig or Fn-like domains. The M-domain may thus function as a spring-like element within cMyBP-C. Supported by NIH HL080367.

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N-Terminal Fragment of Cardiac Myosin Binding Protein-C (CMYBP-C) Reduces Actomyosin Power Output in the Laser Trap Assay

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Mutations in cMyBP-C cause hypertrophic cardiomyopathy, but cMyBP-C's modulation of cardiac contractility is poorly understood. The N-terminus of cMyBP-C binds actin and myosin S2, while the C-terminus binds the myosin rod. Thus, cMyBP-C may act as a tether between the thick and thin filament, imposing a load against which myosin must operate. We used a laser trap force clamp assay (Debold et al. B.J., 2005) to characterize actomyosin's force velocity relationship (F:V) in the absence and presence of a bacterially expressed N-terminal cMyBP-C fragment, C0C3. In this assay a small ensemble of skeletal myosin (~8 heads) translocates a skeletal actin filament under a series of constant loads (1-8 pN), resulting in a classic hyperbolic F:V with a maximum isometric force of 10 ± 2 pN. Adding C0C3 at a 1:1 ratio to myosin depressed actomyosin's F:V: i.e. 48% reduction in maximum unloaded velocity with no effect on maximum force. This reduced actomyosin's maximum power output by 40%. The depressed velocities suggest C0C3 may impose as much as 2 pN of opposing load to the myosin ensemble. As limited numbers of myosin translocate the actin filament under load, a finite probability exists that no myosin will be attached, causing rapid (~2 ms duration) backward movements (34 ± 1 nm) of the actin filament, i.e. slips. The frequency of slips rises with increasing load, which is diminished significantly in the presence of C0C3, suggesting C0C3 prevents slips by its attachment to the actin filament. Based on the depression of the F:V and fewer actin filament slips in the presence of C0C3, we propose that the N-terminus of cMyBP-C can tether actin and impose an effective viscous load in the sarcomere to modulate cardiac power output.

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Charged Amino Acids in the N-Terminus of Cardiac Myosin Binding Protein-C Contribute to Contractile Effects in Permeabilized Myocytes

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Recombinant N-terminal domains of cardiac myosin binding protein C (cMyBP-C) increase calcium-sensitivity of force (pCa50) and the rate of tension redevelopement (k_{tr}) in permeabilized cardiac myocytes. To identify specific amino acids required for these effects, we investigated functional effects of alanine substitution mutations of charged residues near phosphorylation sites within the regulatory M-domain of cMyBP-C. Mutations were introduced into a recombinant protein, C1C2, comprised of the M-domain and its two flanking Ig domains (i.e, C1-M-C2). Compared to wild-type C1C2, a double mutant containing A for R substitutions at positions 270 and 271 upstream of S^{273} (phosphorlyation site A), reduced the ability of C1C2 to increase force in permeabilized rat cardiac trabeculae. Another double mutant, the $^{299}AA^{300}$ substitution for $^{299}KR^{300}$ upstream of S^{302} (phosphorylation site C), also reduced the efficacy of C1C2. A triple mutant that replaced a cluster of three charged residues $^{298}KKR^{300}$ nearly abolished the functional effects of C1C2. These results suggest that positively charged amino acids upstream of protein kinase A phosphorylation sites are important for mediating the contractile effects of C1C2 and that these sites may be involved in the *in vivo* response to -adrenergic stimulation potentially by contributing to binding of the M-domain to actin and/or myosin S2. This work is supported by NIH HL080367 to SPH and a DOD NDSEG fellowship to KLB.

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Uncovering Effects of the Familial Hypertrophic Cardiomyopathy (fHCM) Related ß-Myosin Mutation Arg723gly in Human Cardiomyocytes

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Mutations in sarcomeric proteins have been identified as the major cause of fHCM. The primary functional effects of these mutations at the sarcomere level of cardiomyocytes, however, are still largely unknown. To address this point we studied cardiomyocytes of the left ventricle (LV) from explanted hearts of two patients with ß-myosin (ß-MHC) mutation R723G (Enjuto et al., JMCC 2000).

Our measurements revealed reduced maximum force generation of myocytes but unchanged calcium-sensitivity. In contrast, previous studies on slow skeletal muscle fibers expressing ß-MHC with the same mutation showed reduced calcium-sensitivity and increased maximum force. To address this discrepancy we first determined the expression of mutated and wildtype ß-MHC-mRNA in LV tissue. 62 ± 2% of total ß-MHC-mRNA was found to contain the mutation, just as seen in M. soleus. Secondly, similar to previous findings for failing human heart, gel electrophoresis of the fHCM cardiac tissue showed reduced phosphorylation of troponins I (TnI) and T, myosin binding protein C (MyBP-C), and myosin light chain 2 compared to donor tissue. Adjustment of phosphorylation of TnI and MyBP-C in donor and HCM myocytes by treatment with protein kinase A (PKA), however, uncovered an originally masked reduction in calcium-sensitivity while maximum force was not affected by PKA treatment. Electron microscopy showed reduced myofibrillar density in cardiac tissue samples of the patients which may account for the reduced force. In conclusion, our study reveals that secondary, adaptational processes, triggered by the fHCM-related mutation, can obscure primary effects.

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Zipper-Interacting Protein Kinase Phosphorylates Cardiac Myosin

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Zipper-interacting protein kinase (ZIPK) or DAPK3, is a member of the death-associated-protein kinase family associated with apoptosis in nonmuscle cells where it phosphorylates myosin regulatory light chain (RLC) to promote membrane blebbing. ZIPK mRNA is abundant in muscles, prompting our investigation of its role in the heart. A substrate search on mouse heart homogenates led to the discovery that cardiac RLC is a substrate for ZIPK. Enzyme kinetic studies revealed that both smooth and cardiac RLCs were good substrates, with a Vmax value two-fold greater for cardiac as compared to smooth/nonmuscle RLC. Moreover, ZIPK phosphorylated cardiac RLC at Ser15, the site responsible for modulating Ca^{2+} sensitivity of myofibrillar contraction. Knockdown of ZIPK in isolated neonatal cardiac myocytes by siRNA decreased the extent of RLC Ser15 phosphorylation. Localization studies using adenovirus- mediated overexpression of GFP-ZIPK in neonatal and adult rat cardiac myocytes showed it is in the nucleus, but also the cytoplasm where it may affect RLC phosphorylation. In adult cardiac myocytes, ZIPK appears to associate with myofilaments. ZIPK gene ablation specifically in mouse heart (ZK^{flox}/Nkx2.5Cre) did not affect basal RLC phosphorylation, nor did it induce apparent pathological responses (assessed by histological analysis and heart weight measurements). Based on what is known about the DAPK family, we hypothesize that ZIPK function in the heart may be stress or death signal-dependent. Effects of physiological and pathophysiological stresses on hearts from ZK^{flox}/Nkx2.5Cre mice are currently being investigated. Supported by NIH NHLBI (J. T. S.) and AHA Postdoctoral Fellowship (A.N.C.).

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Kinetics of Mouse Alpha-Myosin S1 a Fast Myosin

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The fast skeletal muscle myosins and the slow/β-cardiac myosins have been well described but few kinetic studies of the α cardiac myosin have been presented. Here we show that the mouse alpha cardiac myosin has more in common with fast skeletal type myosin than the slower B-type myosin. The data for individual rate constant for mouse α myosin S1 are compared to our published values for the rabbit fast muscle and slow/β myosin S1. The ADP affinity for acto.S1 is much weaker (250 μM) than beta-S1 (10 μM) and similar to that of fast rabbit muscle S1 (120 μM). The rate constant for ADP release is too fast to measure for mouse α -S1 and rabbit fast muscle S1 but 63 s^{-1} for the rabbit β-S1. For all myosins affinity of actin form myosin is reduced by ADP and the affinity of ADP for S1 is reduced by actin. This thermodynamic coupling ratio is 10 for β-S1 but ~50-100 for fast muscle S1 and α-S1. The ATP hydrolysis step rate constant estimated from the protein fluorescence changes is 150 s^{-1} similar tor fast muscle S1 (120 s^{-1}) and faster that β-S1 (21 s^{-1}). These values suggest that the ADP release from AMD and the hyrdolysis step are changed in parallel and this is consistent with the hydrolysis step having a role in defining the lifetime of the detached state (M.ATP) while ADP release controls the lifetime of the attached state. Thus the duty ratio can remain similar while the cycle time is altered. All other values assayed show no major difference between the three isoforms of myosin. Overall despite the greater sequence similarity between α and β cardic S1 the α-S1 shares kinetic properties with the fast muscle S1.